Squalamine Treatment of Human Tumors in *nu/nu* Mice Enhances Platinum-based Chemotherapies[1]

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ABSTRACT

Squalamine, an antiangiogenic aminosterol, is presently undergoing Phase II clinical trials in cancer patients. To broaden our understanding of the clinical potential for squalamine, this agent was evaluated in *nu/nu* mouse xenograft models using the chemoresistant MV-522 human non-small cell lung carcinoma and the SD human neuroblastoma lines. Squalamine was studied alone and in combination with either cisplatin or paclitaxel plus carboplatin. Squalamine alone produced a modest MV-522 tumor growth inhibition (TGI) and yielded a TGI with cisplatin that was better than cisplatin alone. Squalamine also significantly enhanced the activity of paclitaxel/carboplatin combination therapy in the MV-522 tumor model. Squalamine similarly improved the effectiveness of cisplatin in producing TGI when screened against the SD human neuroblastoma xenograft. Xenograft tumor shrinkage was seen for the MV-522 tumor in combination treatments including squalamine, whereas no tumor shrinkage was seen when squalamine was omitted from the treatment regimen. To gain a greater understanding of the mechanism by which squalamine inhibited tumor growth in the xenograft studies, *in vitro* experiments were carried out with vascular endothelial growth factor-stimulated human umbilical vein endothelial cells in culture exposed to squalamine. Squalamine treatment was found to retard two cellular events necessary for angiogenesis, inducing disorganization of F-actin stress fibers and causing a concomitant reduction of detectable cell the surface molecular endothelial cadherin (VE-cadherin). We propose that the augmentation by squalamine of cytotoxicity from platinum-based therapies is attributable to interference by squalamine with the ability of stimuli to promote endothelial cell movement and cell-cell communication necessary for growth of new blood vessels in xenografts after chemotherapeutic injury to the tumor.

INTRODUCTION

Lung cancer is the most frequently diagnosed cancer today, with worldwide estimates of new occurrences in excess of a million cases per year (1). In the United States, there are >170,000 new cases of lung cancer per year, with 80–85% of this number being NSCLC[3] (2). Despite the increasing number of newly approved drugs for the treatment of NSCLC, there is a great need for improved treatment of patients with nonresectable NSCLC because 5-year survival rates for these patients are less than 5% (2, 3). The chemotherapeutic basis for treatment of advanced NSCLC has been platinum-based regimens since the late 1980s and early 1990s, when it was demonstrated that cisplatin-containing treatments produced improved 1-year survival rates of 15–25% compared with previously recorded 1-year survival rates of 10–12% using best supportive care (4, 5). Randomized trials at that time also confirmed that cisplatin combined with other therapeutic agents, notably the combination cisplatin/etoposide, generally was superior to the use of other therapeutic agents alone (6–10). Carboplatin, a cisplatin analogue, gained attention when it displayed marginal activity but produced the longest median survival rate in a multi-armed Eastern Cooperative Oncology Group trial (11). Another important agent that has emerged in NSCLC therapy is paclitaxel. Paclitaxel has shown increased response rates and/or improved patient survival when combined with platinum agents for treatment of NSCLC (12–14). Paclitaxel plus carboplatin has been examined in recent years under various conditions of infusion in several Phase I/II or Phase II NSCLC studies and yielded response rates in the range of 20–62% (15). However, paclitaxel/carboplatin has not shown significant improvement over other favored NSCLC combination therapies (cisplatin/etoposide, cisplatin/vinorelbine) in randomized Phase III trials (16, 17), although some oncologists favor the paclitaxel/carboplatin combination for its better patient tolerability and compliance.

Neuroblastoma is a second tumor type for which platinum-based therapy is recommended in certain high-risk patient populations. Neuroblastoma is the most common childhood solid

tumor outside of the cranium. Approximately 70% of neuroblastoma patients present with disseminated disease, and for these patients, the prognosis is generally dismal; the five-year survival rate for children with stage IV neuroblastoma is <20% (18–20). Risk-based management of neuroblastoma patients is now common (21), with chemotherapy recommended for those with localized unresectable or disseminated disease. Platinum agents play an important role in the armentarium of cytotoxic compounds used to treat advanced neuroblastoma. For example, the drug pair cisplatin/teniposide has a clinical outcome equivalent to the older combination of cyclophosphamide and doxorubicin in advanced neuroblastoma (19) and is a standard option for first- or second-line treatment.

Interest in growth-modulating chemotherapies for cancer has grown in the past several years, especially interest in anti-angiogenic compounds that limit or prevent the growth of new blood vessels in tumors (22–24). It is thought that angiogenesis inhibitors are likely to be relatively noncytotoxic, to exhibit a different spectrum of deleterious effects than those caused by the toxic agents presently approved for cancer therapy, and to work well in combination chemotherapy. The importance of certain angiogenic growth factors found elevated in tumors, especially VEGF, has been underscored by positive results with treatments that interfere with VEGF in animal studies (25–27) and in a recent Phase II clinical trial (28). These data suggest that anti-VEGF therapies may hold promise for effective therapy in advanced cancers such as NSCLC. It is hoped that angiogenesis inhibitors that interfere with VEGF expression or VEGF function can have a significant impact on long-term survival in advanced NSCLC as well as in neuroblastoma and other solid tumors when used with cytotoxic platinum-based drug combinations, without the addition of noxious side effects (24, 29).

Squalamine is a natural aminosterol that has been shown to be an angiogenesis inhibitor and an antitumor compound (30–33). In various xenograft models, squalamine has been effective against primary lung (32, 33), breast (32, 34), brain (30), ovarian (35) and prostate (36) tumors and in reducing lung metastases (32). The greatest effect seen with squalamine in preclinical studies has been in combination with the chemotherapeutic agents cyclophosphamide, cisplatin, carboplatin, or 5-fluorouracil (32, 33).

The primary objective of the experiments reported here was to assess squalamine as an inhibitor of growth for tumor xenografts using the chemoresistant human non-small cell lung tumor line MV-522 and the human SD neuroblastoma line, both studied with squalamine in combination with cisplatin, and also the MV-522 cell line in triple therapy with paclitaxel/carboplatin plus squalamine. Our studies support squalamine as being an effective inhibitor for both types of xenografts, notably when combined with chemotherapy treatment. Because changes in the polymerization state of actin or in cell-cell interaction in the endothelium could provide a biochemical basis for the ability of squalamine to inhibit VEGF-driven tumor growth in these studies, we also tested whether or not squalamine affects organization of the endothelial cell actin cytoskeleton and of transmembrane cell-cell adhesion elements connected to the actin cytoskeleton in VEGF-stimulated HUVECs maintained in culture.

MATERIALS AND METHODS

Reagents. Cisplatin (Platinol; lyophilized powder), carboplatin (Paraplatin; 0.9% NaCl solution), and paclitaxel (Taxol®; 50% Cremophor EL solution in ethanol) were purchased from Bristol-Myers Squibb (Princeton, NJ). Chemically synthesized squalamine was prepared by Magainin Pharmaceuticals Inc. TRITC-phalloidin, BSA, and Triton X-100 were from Sigma-Aldrich (St. Louis, MO). A monoclonal antibody against VE-cadherin (Cadherin-5) was obtained from PharMingen (San Diego, CA) and FITC-goat antimouse secondary antibody from Chemicon (Temacula, CA).

Cell Lines. The MV-522 human non-small cell lung adenocarcinoma was derived and maintained in cell culture as described previously (37, 38). The SD neuroblastoma cell line was a gift of Dr. Peter Houghton (St. Jude Children's Research Hospital, Memphis, TN) and was passaged routinely in mice. Both cell lines were derived from female human patients. HUVECs were grown on fibronectin-coated coverslips in supplemented EGM medium (Clonetics Corporation, Walkersville, MD) as described previously (30). Cultured HUVECs were serum starved overnight (>12 h) prior to experimental use.

***In Vivo* Evaluation in Human MV-522 or SD Tumor Xenograft Models.** All of these studies were performed with the prior approval of the Institutional Animal Care Program of The University of Texas Health Science Center at San Antonio. Female Harlan Sprague Dawley (Indianapolis, IN) *nu/nu* mice weighing ~20 g were implanted subcutaneously by trocar with ~1-µl (mm³) fragments of MV-522 human lung or SD neuroblastoma tumors harvested from subcutaneously grown tumors in nude mice hosts. When tumors reached ~5 × 5 mm or about 60 mg in size (12 days after inoculation), the animals were distributed into treatment and control groups with near-equivalent distributions by weight and tumor size. Each group contained 7–10 mice bearing tumors, depending on the tumor type; each mouse was ear-tagged and followed individually throughout the experiment. The administration of drugs or vehicle began the day on which the animals were assigned to groups (day 1). Vehicle in all of the studies was 5% dextrose in water. Drug doses and schedules were initially selected based on prior measurements of maximum tolerated doses for each drug individually or, in the instance of paclitaxel/carboplatin, for both drugs administered together in non-tumor-bearing mice (data not shown). Cisplatin and paclitaxel diluted in 0.9% NaCl were delivered as 2 or 10 mg/kg/day (q.d. for 5 days) doses, respectively, and carboplatin was administered in 0.9% NaCl at 20 mg/kg/day (q.d. for 5 days). Squalamine was given in 5% dextrose in water b.i.d. on day 5 or b.i.d. for days 1–5, 8, and 9 at doses of 0.1, 1, 5, or 10 mg/kg/dose. Dosing b.i.d. was selected because the plasma elimination half-life of squalamine in mice is 1–4 h. All of the drugs were administered by i.p. injection.

Mice were weighed twice weekly until the mean weight of most or all of the treatment groups after drug therapy approximated that of the control group. Tumor measurements were taken by calipers twice weekly starting on day 1 and were converted to milligrams tumor weight by the well-known formula: ½ (width^2 × length) (39, 40). The experiments were terminated when control tumors reached an estimated mean

Table 1 Effect of squalamine and cisplatin or paclitaxel plus carboplatin on the growth of the MV-522 human lung tumor xenograft in nude mice

Each treatment group consisted of 10 mice. The percentage TGI was calculated using the final mean excised tumor weight for each treatment group rather than the final tumor weights estimated by dimensional measurement. The squalamine dose as listed was administered on a daily basis as a split dose.

Group	Dose (mg/kg/day)	Route & schedule	Body weight change (day 24)	Final excised tumor weight (mg; mean $\pm$ SE) (TGI)	% TGI	Mice with partial shrinkage	Mean % tumor shrinkage	No. of toxic deaths
Control	Vehicle	i.p.: b.i.d. days 1–5, 8–9	+4.3%	758.6 $\pm$ 103.5		0		0
Squalamine	20	i.p.: b.i.d. days 1–5, 8–9	+5.3%	613.6 $\pm$ 64.6	28.6%	0		0
Cisplatin	2.0	i.p.: q.d. $\times$ 5[a]	+7.9%	218.7 $\pm$ 44.2	73.3%	2	41.6%	0
Squalamine	20	i.p.: b.i.d. days 1–5, 8–9						
+ cisplatin	2.0	i.p.: q.d. $\times$ 5	−0.9%	105.0 $\pm$ 28.6	86.9%	3	45.1%	3
Paclitaxel	10	i.p.: q.d. $\times$ 5						
+ carboplatin	20	i.p.: q.d. $\times$ 5	+7.0%	365.9 $\pm$ 66.0	60.3%	0		0
Paclitaxel	10	i.p.: q.d. $\times$ 5						
+ carboplatin	20	i.p.: q.d. $\times$ 5						
+ squalamine	20	i.p.: b.i.d. days 1–5, 8–9	+0.0%	56.4 $\pm$ 14.5	96.1%	4	54.8%	3

[a] Days 1–5, 8–9 refers to dosing on days 1 through 5 and 8 through 9, inclusive, after initiating chemotherapeutic treatment.
[b] $\times$5, for 5 days.

tumor size of 1 g. All of the mice were then weighed and killed, and their tumors were excised. Excised tumors were weighed prior to their fixation in 10% formalin, and the mean excised tumor weight growth or increase from day 1 values for each treatment group was calculated for all of the animals surviving until the scheduled terminal sacrifice. In these xenograft models, the [mean excised treated-tumor weight increase (T) divided by the mean excised control-tumor weight increase (C) = T/C] $\times$ 100% was subtracted from 100% to give the TGI value for each group. Mice with a reduction in tumor size at the end of the experiment relative to the initial tumor size were not included in the calculation of TGI. Consequently, TGI values represent minimal estimates of tumor response to chemotherapy.

Some drug combinations caused shrinkage of some tumors in the MV-522 tumor xenograft model. With these combinations, the final excised weight of a given tumor was subtracted from its own calculated weight at the start of treatment on day 1 for the relevant mice (39, 40). This difference divided by the initial tumor weight times 100% was the percentage tumor shrinkage during the experiment. A mean percentage tumor shrinkage was then calculated from the data if more than one mouse in a group experienced a reduction in tumor size as determined at experiment termination. If the tumor completely disappeared in a mouse by the time of scheduled terminal sacrifice, this was considered a complete response or complete tumor shrinkage.

The toxicities of the treatment regimens were estimated by following changes in animal body weight and the incidence of drug-associated deaths.

A one-way ANOVA with subsequent pairwise comparisons by Bonferroni's *t* test was used for statistical analysis of final excised tumor weight changes for homoscedastic tumor weights with normal distributions. Analyses of tumor weights that had nonnormal distributions and/or were heteroscedastic were carried out using a Kruskal-Wallis statistical test, with a Mann-Whitney rank sum test for pairwise comparisons. Differences between means or ranks as appropriate were considered significant when yielding a $P < 0.05$.

Immunohistochemical Double Staining of F-Actin Stress Fibers and VE-Cadherins. HUVECs grown on glass coverslips were stimulated with 20 ng/mL VEGF and treated with 20 μM of squalamine for 2 h, whereas VEGF-stimulated controls were treated only with vehicle (0.1% DMSO). This concentration of squalamine was selected based on a pharmacokinetic study injecting 10 mg/kg squalamine i.p. (the maximum dose given mice b.i.d. in the xenograft studies described in this paper) that revealed the level of squalamine in the rodent plasma was 10–20 μM after 12 h. The treated cells were fixed with 3.7% formaldehyde (pH 7.5) for 5 min followed by three washes with Tris-HCl buffered saline. Fixed cells were permeabilized at least 1 h at 4°C in 0.1% Triton X-100, 0.1% BSA in PBS. The coverslips were then incubated for 2 h at room temperature with a 1:100 dilution of murine anti-VE-cadherin monoclonal antibody before rinsing them in PBS and incubating them with a 1:400 dilution of FITC-goat antimouse antibody and 250 ng/ml TRITC-phalloidin for 1 h at room temperature. The stained coverslips were washed in PBS and stored in the dark until examination by fluorescence microscopy.

RESULTS

Squalamine and Cisplatin: MV-522 Human Lung Tumor. Squalamine alone, when given at a dose of 20 mg/kg/day on days 1–5 and 8–9 to animals bearing the MV-522 tumor, produced a modest TGI value of 28.6% (Table 1). Single-agent cisplatin (2 mg/kg/day, q.d. for 5 days) was substantially active and produced a TGI of 73.3% at terminal sacrifice (Table 1). The addition of squalamine to cisplatin treatment further improved control of MV-522 tumor growth. Squalamine plus cisplatin yielded a mean excised tumor size of 105.0 $\pm$ 24.0 mg (which corresponds to a mean tumor growth of 48.8 $\pm$ 32.9 mg during the experiment) and a TGI of 86.9% relative to control tumor growth for all of the mice surviving to terminal sacrifice. Two mice in the cisplatin treatment group displayed smaller tumors at the end of the experiment compared with day 1, with a mean tumor shrinkage of 41.6%, and three
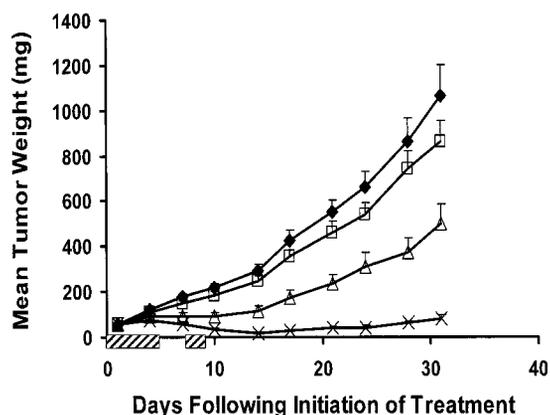
Fig. 1 Effect of combination chemotherapy with and without squalamine cotreatment. Nude mice bearing the MV-522 human lung tumor were treated for days 1–5 with vehicle (5% dextrose in water; ◆) or with paclitaxel (10 mg/kg/day, q.d. for 5 days) and carboplatin (20 mg/kg/day, q.d. for 5 days; △, ×). Some treatment groups (□, ×) received squalamine (20 mg/kg/day) on days 1–5 and 8–9. *Error bars*, SE. ▨, squalamine dosing period.

mice in the squalamine-plus-cisplatin treatment group had smaller tumors (mean tumor shrinkage of 45.1% for these animals). None of the mice had complete tumor shrinkage.

Dosing of squalamine in this initial experiment was stopped on day 9 in all of the treatment groups when a cumulative total of three animal deaths had occurred in the squalamine-plus-cisplatin experimental group. The nadir of body weight loss in all of the treatment groups was observed on day 7, and all of the treatment groups except squalamine plus cisplatin had mean body weights similar to controls by the last day of animal weighing (day 24).

Squalamine plus Paclitaxel and Carboplatin: MV-522 Human Lung Tumor. Paclitaxel at 10 mg/kg, q.d. for 5 days, in combination with carboplatin at 20 mg/kg, q.d. for 5 days, was tolerable to the *nu/nu* mice and represented the maximal combination dosing that was not toxic. Each drug is known to cause reduction in MV-522 tumor growth rate at these doses when given i.p. on a daily basis, q.d. for 5 days (37, 39, 41). The combination of paclitaxel and carboplatin with this dosing regimen in the MV-522 human lung tumor xenograft model produced a mean excised tumor weight of 365.9 ± 66.0 mg at day 31, corresponding to a TGI of 60.3%. By contrast, the TGI index for the squalamine/paclitaxel/carboplatin arm of this study, using squalamine at a dose of 20 mg/kg/day on days 1–5 and 8–9, was 96.1% (Table 1). The benefit of chemotherapy on tumor size became apparent by day 7, and differences in mean tumor sizes between the two paclitaxel and carboplatin-containing arms of this study continued to increase thereafter (Fig. 1). No mouse receiving paclitaxel plus carboplatin was noted to undergo tumor shrinkage by the end of the experiment, but four mice in the triple combination chemotherapy cohort displayed tumor shrinkage, with a mean reduction in tumor size for these animals of 54.8% (Table 1).

Three mice in the cohort treated with squalamine/paclitaxel/carboplatin (20/10/20 mg/kg/day) died on day 7, 11 or 12. These results suggested that the tumor reduction seen in com-

bination treatments that include squalamine might be a toxic effect of squalamine. To rule out toxicity as the cause for the effectiveness of squalamine as an antitumor compound, a dose ranging experiment with the MV-522 lung tumor xenograft model was undertaken with paclitaxel and carboplatin using squalamine at doses of 0.2–20 mg/kg/day on days 1–5 and 8–9. All of the doses of squalamine except the lowest squalamine dose (0.2 mg/kg/day) studied in conjunction with carboplatin and paclitaxel reduced the rate of tumor growth compared with carboplatin plus paclitaxel alone (Table 2; Fig. 2). The mean excised tumor growths for squalamine-treated groups combined with carboplatin and paclitaxel were 132.0 ± 46.4 mg (TGI, 74.2%) at a daily squalamine dose of 0.2 mg/kg ($P > 0.05$ compared with carboplatin plus paclitaxel) and less than 77.0 mg (TGI, ≥78.8%) at 2, 10, or 20 mg/kg/day squalamine ($P < 0.05$ compared with carboplatin plus paclitaxel). The maximal enhancement seen with squalamine in combination with carboplatin and paclitaxel occurred with 2 or 10 mg/kg/day dosing. The daily dosage of 10 mg/kg/day squalamine with carboplatin/paclitaxel was not quite as effective as 2 mg/kg/day, but the difference was negligible. The advantage of adding squalamine to paclitaxel and carboplatin treatment of the MV-522 tumor was apparent at all of the doses by day 5 and persisted throughout the course of the experiment (Fig. 2).

There were several mouse deaths that occurred with triple chemotherapy during the dose ranging experiment; one mouse died receiving 10 mg/kg/day squalamine, and three mice died receiving 20 mg/kg/day squalamine. However, no mice died at the optimal squalamine dose of 2 mg/kg/day, and weight changes in both the 0.2 and 2 mg/kg/day squalamine treatment groups were not significantly different from controls at any time during the experiment, which indicated that the antitumor activity of squalamine is likely to be independent of systemic toxic effects. It was further observed that no mice receiving paclitaxel and carboplatin alone had tumor shrinkage. By contrast, addition of squalamine to paclitaxel/carboplatin caused tumor shrinkage in at least two mice in each squalamine-treated combination chemotherapy group (Table 2).

The persistence of the effect of squalamine on tumor growth more than 3 weeks after the last dosing with squalamine (Figs. 1 and 2) led us to investigate the period of time over which a total squalamine dose was given. MV-522 tumor growth in mice was determined for squalamine given b.i.d. as a 1-day dose of 0.2–20 mg/kg combined with paclitaxel and carboplatin. There was a squalamine dose-related enhancement of TGI for paclitaxel plus carboplatin with single daily squalamine dosing (Fig. 3), with the best responses seen with 10 and 20 mg/kg squalamine (Table 2). Tumor shrinkage was again seen in at least two mice in each squalamine/paclitaxel/carboplatin-treated cohort, but no long-term reduction in tumor size was seen with any mice treated only with paclitaxel plus carboplatin. There also was one complete tumor shrinkage observed in a mouse that received 20 mg/kg squalamine on day 5 along with paclitaxel and carboplatin. The mean percentage tumor shrinkage at terminal sacrifice in the treatment groups for which tumor shrinkage occurred was in the range of 21.0–50.4%.

Table 2 Effect of varying single or multiple daily doses of squalamine combined with paclitaxel plus carboplatin on growth of the MV-522 human lung tumor xenograft in nude mice

Each treatment group consisted of 10 mice. The % TGI was calculated using the final mean excised tumor weight for each treatment group rather than the final tumor weights estimated by dimensional measurement. The squalamine dose as listed was administered on a daily basis as a split dose.

Group	Dose (mg/kg/day)	Route & schedule	% weight change (day 32)	Final excised tumor weight (mg) (mean ± SE)	% TGI	Mice with tumor shrinkage (day 32)	Mean % tumor shrinkage	Mice with complete shrinkage
Control	Vehicle	i.p.: b.i.d. days 1–5, 8–9	+7.9%	739.2 ± 69.4		0		0
Paclitaxel	10	i.p.: q.d. × 5[a]						
+ carboplatin	20	i.p.: q.d. × 5	+8.6%	288.5 ± 76.4	66.9%[b]	0		0
Paclitaxel	10	i.p.: q.d. × 5						
+ carboplatin	20	i.p.: q.d. × 5						
+ squalamine	0.2	i.p.: b.i.d. day 5	+6.1%	263.0 ± 81.7	70.7%[b]	2	46.4%	0
Paclitaxel	10	i.p.: q.d. × 5						
+ carboplatin	20	i.p.: q.d. × 5						
+ squalamine	2.0	i.p.: b.i.d. day 5	+8.6%	274.2 ± 91.2	69.0%[b]	2	50.4%	0
Paclitaxel	10	i.p.: q.d. × 5						
+ carboplatin	20	i.p.: q.d. × 5						
+ squalamine	10	i.p.: b.i.d. day 5	+11.9	176.9 ± 49.7	83.2%[b]	3	21.0%	0
Paclitaxel	10	i.p.: q.d. × 5						
+ carboplatin	20	i.p.: q.d. × 5						
+ squalamine	20	i.p.: bid day 5	−11.9%	161.7 ± 64.6	85.7%[b]	4	45.1%	1
Paclitaxel	10	i.p.: q.d. × 5						
+ carboplatin	20	i.p.: q.d. × 5						
+ squalamine	0.2	i.p.: bid days 1–5, 8–9	+8.3%	195.8 ± 40.4	80.4%[b]	2	55.2%	0
Paclitaxel	10	i.p.: q.d. × 5						
+ carboplatin	20	i.p.: q.d. × 5						
+ squalamine	2.0	i.p.: b.i.d. days 1–5, 8–9	+12.6%	88.0 ± 18.9	96.6%[c]	4	46.9%	0
Paclitaxel	10	i.p.: q.d. × 5						
+ carboplatin	20	i.p.: q.d. × 5						
+ squalamine	10	i.p.: b.i.d. days 1–5, 8–9	+4.7%	98.4 ± 28.5	94.9%[c]	5	41.3%	0
Paclitaxel	10	i.p.: q.d. × 5						
+ carboplatin	20	i.p.: q.d. × 5						
+ squalamine	20	i.p.: b.i.d. days 1–5, 8–9	+0.5%	142.1 ± 37.9	88.6%[c]	3	17.8%	0

[a] × 5, for 5 days.
[b] $P < 0.05$ relative to control.
[c] $P < 0.05$ relative to carboplatin/paclitaxel group.

Squalamine plus Cisplatin: SD Human Neuroblastoma Tumor. Treatment of another human tumor xenograft, the SD human neuroblastoma, responsive to a platinum compound was carried out to further evaluate the ability of squalamine to enhance platinum-mediated TGI. Squalamine was slightly active as a single agent when given to mice bearing the SD human neuroblastoma xenograft; squalamine produced a TGI of 23.7% when given b.i.d. at 10 mg/kg/day (Table 3). Single agent cisplatin (2 mg/kg, q.d. for 5 days) also was slightly active with the SD tumor, producing a TGI of 22.6% at terminal sacrifice. Squalamine plus cisplatin yielded a mean excised tumor weight in the range of 233.0–432.1 mg for the two squalamine doses tested, corresponding to an improved TGI of 45.3–75.8% relative to control tumor growth (Table 3). No tumor shrinkages were observed in any treatment group, nor were any animal deaths observed, but temporary mean body weight losses were observed in all of the treated animals except for the low-dose-squalamine group in comparison with animal weights on day 1.

Squalamine Influence on HUVEC F-Actin Stress Fibers and VE-Cadherin. VE-cadherin is a member of the cadherin family involved in cell-cell adherens junction maintenance (42, 43) and is associated with actin filament attachment sites on the cell surface that modulate cell activation and proliferation. We found that squalamine disrupted polymeric F-actin fibers of VEGF-stimulated HUVECs in culture (Fig. 4). At the same time and for the same cultures, much less VE-cadherin is detectable at the endothelial cell-cell junction after squalamine treatment (Fig. 4). However, there is a corresponding increase in the amount of VE-cadherin found perinuclear, suggestive of a squalamine-induced internalization process that takes VE-cadherin off the membrane and moves it with limited degradation into an intracellular compartment. Inhibitory effects on actin fibrils and VE-cadherin display also are seen with unstimulated subconfluent HUVECs or those pretreated with thrombin (data not shown) after exposure to squalamine.

DISCUSSION

The chemotherapy of advanced or unresectable NSCLC and neuroblastomas remains a major issue in cancer chemotherapy. Platinum-based therapies have been proven slightly effective in the past 15 years for both types of tumors. The combination of carboplatin and paclitaxel has seen increased usage in NSCLC, but mixed clinical results with carboplatin plus paclitaxel (44, 45) have been reported from large multicenter Phase III randomized trials in the last 2 years. Carboplatin plus paclitaxel has also been shown to be a more costly treatment than the clinically equivalent cisplatin/vinorelbine combination (46).
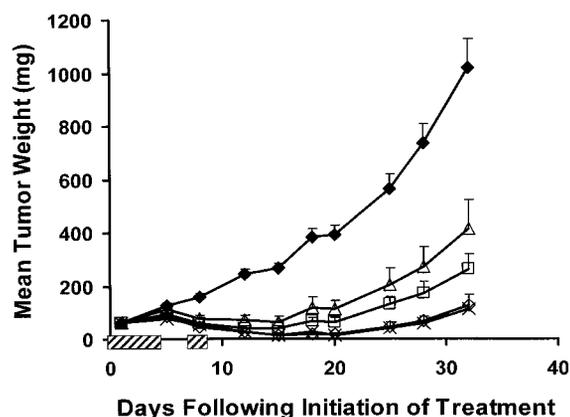
Fig. 2 MV-522 tumor growth after treatment with vehicle (♦), paclitaxel plus carboplatin (10 and 20 mg/kg/day, respectively, q.d. for 5 days) alone (△) or paclitaxel plus carboplatin combined with daily doses of 0.2 mg/kg (□), 2.0 mg/kg (×), or 10.0 mg/kg (◇) squalamine on days 1–5 and 8–9. Treatment with paclitaxel and carboplatin ended on day 5 and with squalamine on day 9. *Error bars*, SE. ▨, squalamine dosing period.

Fig. 3 MV-522 tumor growth after treatment with paclitaxel plus carboplatin (10 and 20 mg/kg/day, respectively, q.d. for 5 days) alone (♦) or combined with 1-day doses of 0.2 mg/kg (□), 2.0 mg/kg (△), 10.0 mg/kg (◇), or 20.0 mg/kg (×) squalamine administered b.i.d. on day 5. Treatment with paclitaxel and carboplatin ended on day 5; squalamine was administered only on day 5. *Error bars*, SE. ▨, day of squalamine dosing.

There is, therefore, an acknowledged need for additional changes for the better in NSCLC therapy, especially because new active agents such as vinorelbine, paclitaxel, and gemcitabine have provided only minor improvements in survival for disseminated disease in NSCLC patients (3, 47, 48). Although not as significant a public health problem as lung cancer, neuroblastoma is a devastating central nervous system disease. Cisplatin-containing chemotherapy is frequently used for treatment of intermediate-to-high-risk neuroblastomas, but metastatic neuroblastoma patients still have a poor response rate. Improvements in treatment of disseminated neuroblastoma are clearly required.

Squalamine in combination with cisplatin or with paclitaxel plus carboplatin enhances control of growth for both the MV-522 human lung tumor and the human SD neuroblastoma tumor as xenografts when compared with the cytotoxic agents alone. We have previously provided evidence that squalamine has specificity for endothelial cells and is thought to effectively drive activated endothelial cells into quiescence by a mechanism still to be fully elucidated (30, 31). Not all endothelial cells appear to be sensitive to squalamine, however. The most sensitive cells appear to be those found in newly emerging or embryonic microvessels, vasculature that lacks accessory stromal cells, and extracellular matrix elements characteristic of mature blood vessels (30). Embryonic tumor endothelium is characteristic of growing vessels in tumors soon after a population of tumor cells has been killed (27). We consequently designed our MV-522 and SD tumor studies to provide squalamine treatment concurrent with initiating tumor cell kill with cisplatin or carboplatin plus paclitaxel.

The addition of squalamine to platinum-based dosing improved the response of MV-522 and SD tumors to chemotherapy. However, there also appeared to be an increased toxicity when squalamine was used at high doses in combination. There is no evident overlap in toxicity for squalamine with platinum compounds and th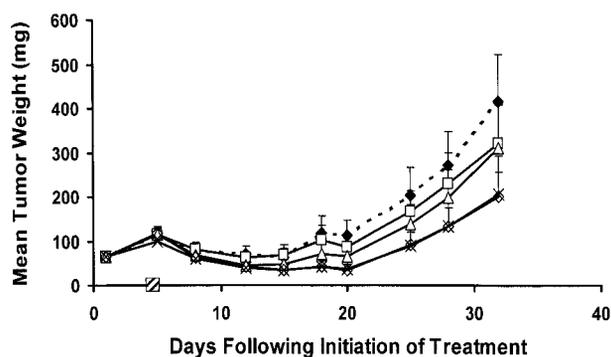e deleterious interaction of these agents and squalamine requires further study. It is unlikely that toxic effects of squalamine alone are the basis for the enhancement of cytotoxic therapy with squalamine based on our obtaining significant increases in MV-522 tumor responses with squalamine at nontoxic doses and on demonstrating that animal body weight loss after squalamine plus cytotoxic chemotherapy is transient and fully reversible.

We tested squalamine in combination with paclitaxel and carboplatin for the MV-522 lung tumor because these two drugs have become a recognized drug combination for treating NSCLC patients. Although paclitaxel and carboplatin together did cause temporary tumor shrinkage in some of the treated animals bearing the MV-522 tumor, an inhibition of tumor growth was not seen in any paclitaxel/carboplatin-treated tumor by the time control tumors had reached 1 g in size. By contrast, mice treated with squalamine doses as low as 0.2 mg/kg/day in combination with paclitaxel and carboplatin experienced substantial tumor shrinkage that was still evident 3 weeks after completion of squalamine treatment.

Squalamine had a more modest impact on human SD neuroblastoma TGI when combined with cisplatin compared with the effect of squalamine plus cisplatin on MV-522 lung tumor growth. This result may reflect the fact that the SD neuroblastoma is less responsive to chemotherapy treatment than the MV-522 lung tumor. Alternatively, there may be a component of direct lung tumor cell sensitivity to squalamine that is not present with the SD neuroblastoma cell line. Careful titration of dose for cytotoxic chemotherapies and for squalamine may be necessary to optimize the effectiveness of squalamine in humans with regard to a particular tumor type, as was done in our exploration of squalamine treatment of MV-522 lung tumor xenografts.

The mechanistic basis for the enhancement of cytotoxic chemotherapy with squalamine seen in the xenograft mouse models is not clear. Growth factors such as VEGF and cytokines released by tumor and stromal cells cause formation and reorganization of F-actin stress fibers in endothelial cells as part of their programming of the cytoskeleton to allow endothelial cell

Table 3 Effect of varying doses of squalamine combined with cisplatin on growth of the SD human neuroblastoma xenograft in nude mice

The % TGI was calculated using the final mean excised tumor weight for each treatment group rather than the final tumor weights estimated by dimensional measurement. The squalamine dose as listed was administered on a daily basis as a split dose.

Group	n	Dose (mg/kg/day)	Route & schedule	Weight change (day 24)	Final excised tumor weight (mg; mean ± SE)	% TGI
Control	7	Vehicle	i.p.: b.i.d. × 5[a]	+7.1%	731.1 ± 189.5	
Squalamine	8	1.0	i.p.: b.i.d. × 5	+9.2%	737.3 ± 198.1	
Squalamine	8	10	i.p.: b.i.d. × 5	+7.8%	573.0 ± 148.9	23.7%
Cisplatin	8	2.0	i.p.: q.d. × 5	+7.8%	580.5 ± 135.3	22.6%
Cisplatin + squalamine	8	2.0 1.0	i.p.: q.d. × 5 i.p.: b.i.d. × 5	+12.8%	432.1 ± 110.2	45.3%
Cisplatin + squalamine	8	2.0 10	i.p.: q.d. × 5 i.p.: b.i.d. × 5	−3.2%	233.0 ± 47.3	75.8%

[a] × 5, for 5 days.
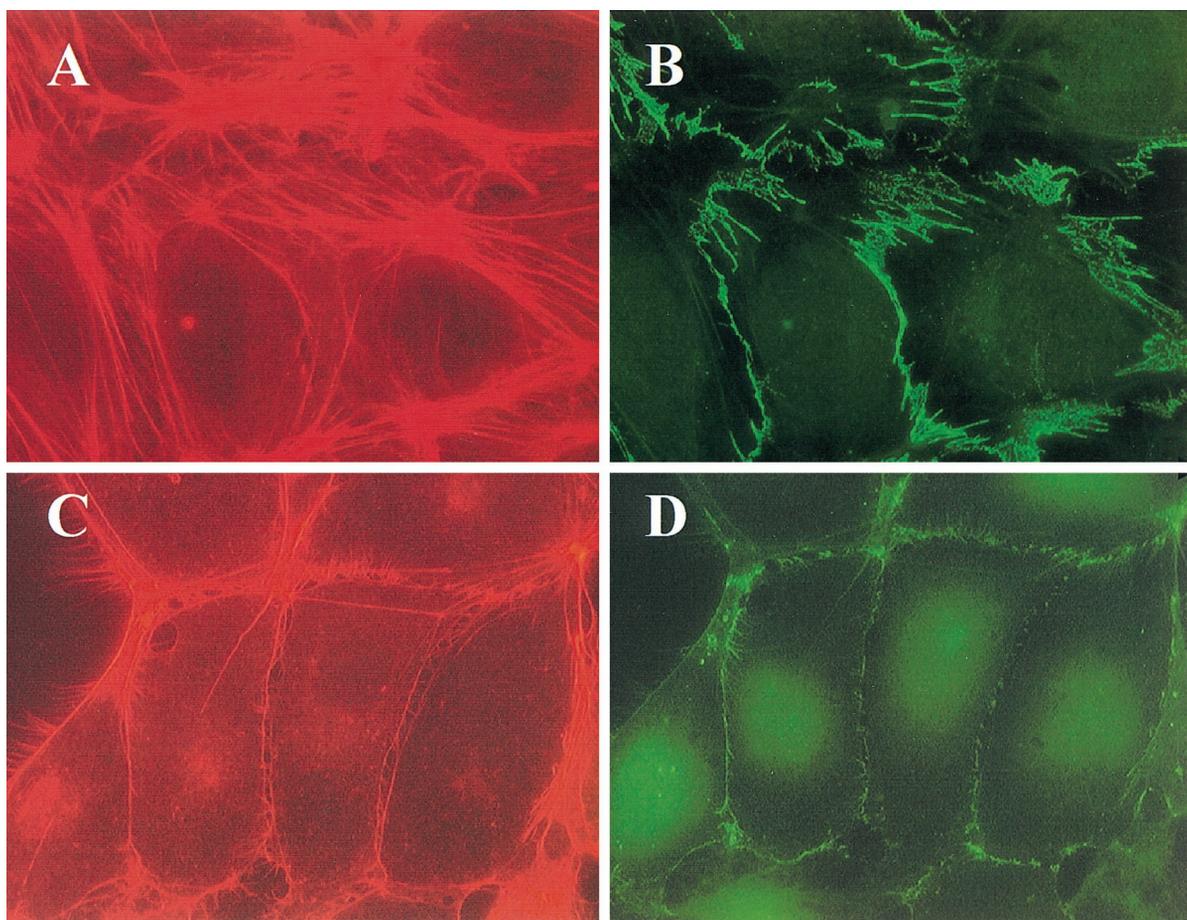
Fig. 4 The effect of squalamine on F-actin stress fibers and VE-cadherin expression in HUVECs. HUVECs grown on coverslips were exposed to medium containing 20 ng/mL VEGF without (*A, B*) or with (*C, D*) 20 μM squalamine treatment for 2 h before the cell cultures were immunolabeled to visualize F-actin filaments (A, C) or membrane VE-cadherin (B, D). The cells photographed for *A* and *B* are the same, as are those for *C* and *D*.

movement and proliferation during tumor angiogenesis. Cells of the endothelium must also communicate to form newly organized, growing blood vessels and do so through F-actin fiber bundles that link adjacent cells using protein assemblies containing externally displayed VE-cadherin. Blockade or suppression of these protein assemblies in tumor blood vessels might be expected to limit the growth or regrowth of blood vessels after chemotherapeutic damage to tumor cells and tumor endothelium.

On the basis of the *in vitro* data presented in this report, we conclude that squalamine perturbs both actin polymerization and cell-cell adhesion in endothelial cells. Actin depolymeriza-

tion has previously been shown to weaken cell-cell adhesion and potentially disperse VE-cadherin (49); therefore, a cause-effect relationship may exist to explain why squalamine may effect both endothelial cell functions. However, without further investigation, it is not possible to establish whether or not the effect of squalamine on these two cellular phenomena are independent or interdependent.

Our observations on HUVEC responses to squalamine support a concept that squalamine stops new blood vessel growth after platinum-based chemotherapy by disrupting endothelial cell architecture and the integrity of endothelial cell-cell attachment in tumors and, consequently, slows or prevents further tumor growth. Cytostatic or cytocidal blockade of endothelium response by squalamine, therefore, is complementary to antitumor treatments, as was seen in our experiments. We cannot rule out the possibilities that squalamine may alter the *in vivo* pharmacokinetics of cytotoxic agents in mice or that squalamine may inhibit survival factors such as multidrug resistance protein pumps in endothelial cells or tumor cells. However, the ability of squalamine to interfere with expression or maintenance of the cell adhesion molecule VE-cadherin on the endothelial cell surface and to damage the actin cytoskeleton will decrease the ability of the endothelial cells to move, grow, and form new blood vessels and would be expected to occur *in vivo*. Squalamine obstruction of these endothelial cell functions consequently may be a key factor in the ability of squalamine to retard or prevent adequate nourishment of tumors during their regrowth after a chemotherapeutic insult, resulting in tumor growth stasis or tumor regression. This proposed mechanism is persuasive, but more detailed *in vitro* and *in vivo* experimentation will be necessary to resolve the relative contribution of each hypothesized mechanism explaining the positive influence of squalamine on the effectiveness of platinum-based chemotherapy. In particular, any further explication of the mechanism of action for squalamine in combination chemotherapy will require a closer look at generalized signal transduction in endothelial cells because squalamine inhibits the action of multiple growth factors in addition to VEGF (30), which suggests that the ability of squalamine to inhibit functions of the endothelial cell cytoskeleton is a fundamental property of squalamine.

From a signal transduction perspective, both polymerization of F-actin and VE-cadherin display, as stimulated by growth factors or cytokines in endothelial cells, are calmodulin-dependent (50, 51), and changes in calcium/calmodulin signaling could reduce or block cell activation mediated by these cellular changes. In this regard, biochemical investigation of the effect of squalamine on endothelial cells has recently provided evidence that squalamine can bind the calcium-binding mediator calmodulin and inhibit calcium/calmodulin-dependent signal transduction.[4] VEGF-induced proliferation of endothelial cells also requires the action of protein kinase C for angiogenesis (52, 53), some forms of which are calcium-sensitive, but up-regula-

tion of VE-cadherin expression and actin polymerization can also occur as a result of calcium-independent cell signaling circuits involving small GTPases like Rac and Rho (54–56). It, therefore, remains to be shown to what extent squalamine-calmodulin binding is a sufficient or necessary step in squalamine's inhibition of VE-cadherin expression and actin polymerization and how this may be related to the antitumor activity of squalamine.

Our xenograft results provide support for the contention that squalamine may be effective as an adjunct to platinum-based treatment for advanced NSCLC or childhood neuroblastoma. This concept is presently being tested in a Phase II clinical trial with squalamine for stage IIIb/IV NSCLC patients. Similar clinical studies for neuroblastoma patients combining squalamine and cisplatin are in the planning stage.

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